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THE ELECTROPHILE COUNTERATTACK RESPONSE: PROTECTION AGAINST NEOPLASIA AND TOXICITY

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INTRODUCTION

Neoplasia is one important manifestation of genotoxicity, and commonly arises from the interaction of exogenous or endogenously-generated electrophiles with critical nucleophilic centers of DNA. Oxidative cycling, which generates reactive oxygen species (superoxide, peroxide, and hydroxyl radicals), also contributes to DNA damage and to the development of malignancy. Both electrophiles and reactive oxygen species deplete intracellular glutathione and thereby aggravate these toxicities. Hence, it is not surprising that cells have developed elaborate mechanisms to counteract these dangers.

Exposure of animals or their cells in culture to low but tolerated concentrations of electrophiles evokes a series of characteristic and wide-ranging metabolic responses including the coordinated induction (by enhanced transcription) of a number of Phase 2* enzymes that detoxify electrophiles, and the elevation of intracellular levels of reduced GSH (by accelerated synthesis). These responses reduce the susceptibility of cells to higher, potentially hazardous, or even lethal, concentrations of the same or other toxic electrophiles. We suggest that this constellation of protective metabolic responses be designated as: "*Electrophile Counterattack*".

Abbreviations and definitions: Ah receptor, Aryl hydrocarbon receptor; BHA, 2(3)-*tert*-butyl-4-hydroxyanisole; CD, concentration of a compound that doubles the specific activity of quinone reductase in Hepa 1c1c7 cells under specified conditions; GSH, reduced glutathione; QR, quinone reductase, NAD(P)H:(quinone acceptor) oxidoreductase (EC 1.6.99.2); GST, glutathione transferase (EC 2.5.1.18).

*Enzymes involved in the metabolism of xenobiotics have been classified into two broad categories. Phase 1 enzymes (principally cytochromes P-450) functionalize compounds largely by oxidative or reductive reactions, whereas Phase 2 enzymes carry out the conjugations of such functionalized compounds with endogenous ligands (e.g., glutathione and glucuronic acid). Quinone reductase is classified as a Phase 2 enzyme because it serves protective functions, is induced coordinately with other Phase 2 enzymes, and is regulated by enhancer elements that are similar to those controlling Phase 2 enzymes (6).

Analysis of the anticarcinogenic mechanisms of BHA identified the important protective role of the electrophile counterattack response. It was found that administration of BHA to rodents increased the specific activities of glutathione transferases, UDP-glucuronosyltransferases, epoxide hydrolase, NAD(P)H:quinone reductase, and other Phase 2 enzymes in the liver and peripheral tissues of these animals, and also raised levels of reduced GSH (1-4). The activities of Phase 1 enzymes that are regulated by the *Ah* receptor (i.e. cytochromes P-450 IA1 and IA2) were largely unaffected by BHA treatment (5). BHA was thus classified as a *monofunctional* inducer, in contrast to *bifunctional* inducers (e.g., polycyclic aromatics, azo dyes, flavonoids), which elevate both Phase 1 and Phase 2 enzymes (5). These findings led to the formulation of the now widely held view that elevation of Phase 2 enzymes is mainly responsible for the protective effects of BHA against neoplasia and other forms of electrophile toxicity (7). Many lines of evidence attest to the validity of this conclusion. Indeed several novel anticarcinogens have been identified on the basis of their ability to evoke Phase 2 enzyme inductions (8-10).

This paper describes the biological scope of the electrophile counterattack response, the chemistry of the inducers, and the molecular regulation of the response (6).

BIOLOGICAL SCOPE OF THE ELECTROPHILE COUNTERATTACK RESPONSE

Induction of phase 2 enzymes. The earliest and up to now most complete information on the biochemistry of the electrophile counterattack response has been obtained on animals and cells treated with BHA or *tert*-butylhydroquinone which is presumed to be its active metabolite. Feeding of 0.5-1.0% BHA in the diet to mice or rats for 5-7 days profoundly reduced the conversion of benzo(a)pyrene to mutagenic metabolites (1). The livers and peripheral tissues of these animals showed large increases in the specific activities of GSTs (2, 4) and their cognate mRNAs (11-13). Indeed, these normally rather abundant enzymes increased even further and became the most prominent proteins on two-dimensional electrophoresis of mouse liver cytosols (11). Subsequent studies revealed that the elevated GSTs belonged to the α , μ , and π families and that the inductions showed considerable tissue specificity (12, 14-16).

Further studies demonstrated that the capability of chemoprotective agents to elevate enzymes of xenobiotic metabolism was not limited to BHA and was a widespread property of many chemoprotectors. Moreover, the variety of enzymes induced was diverse (7), and included UDP-glucuronosyltransferases, NAD(P)H:(quinone acceptor) oxidoreductase, epoxide hydrolase and a number of additional Phase 2 enzymes (3, 4, 17, 18). A comprehensive review of the protective effects of BHA and

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other synthetic antioxidants, their biological effects, and enzyme induction patterns has been provided by R. Kahl (19).

Typical changes in the activities of Phase 2 enzymes in the livers of mice treated with BHA are shown in Figure 1A. From 5- to 10-fold increases in specific activities of GST, QR, epoxide hydrolase, and UDP-glucuronosyltransferases were observed. In contrast, several cytochrome P-450-related functions (total cytochrome P-450 hemoprotein, aniline hydroxylase, and aminopyrine demethylase) were unaffected or even depressed by such treatment. Based on the marked changes in Phase 2 enzymes in response to BHA administration, it was suggested that

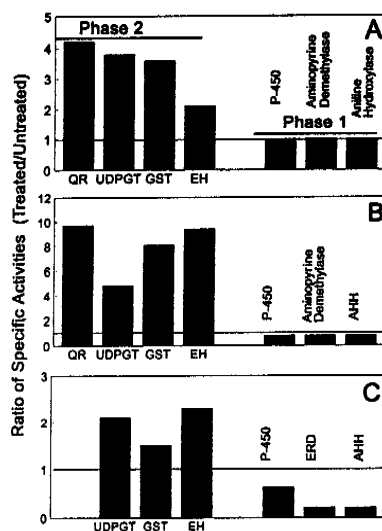


FIG. 1. Effect of monofunctional inducers on specific activities of Phase 1 and Phase 2 enzymes in rodent livers, expressed as the ratios of specific activities of cytosols or microsomes of treated to untreated animals. (A) Feeding of BHA to female CD-1 mice (from Refs (2-4, 17, 18)). (B) Feeding of 1,2-dithiole-3-thione to male F 344 rats (from Ref. (21)). (C) Intraperitoneal administration of bromobenzene to male Sprague-Dawley rats (from Ref. (22)). Note the large increases in the specific activities of glutathione transferases (GST), quinone reductase (QR), UDP-glucuronosyltransferases (UDPGT), and epoxide hydrolase (EH). Also note the lack of increase (or possibly decline) in the total cytochrome P-450 content (P-450), and aminopyrine demethylase, aniline hydroxylase, aryl hydrocarbon hydroxylase (AHH), and ethoxyresorufin O-deethylase (ERD) activities.

BHA might block chemical carcinogenesis by enhancing the detoxication capacity of tissues for the ultimate carcinogenic electrophiles, and that the balance between Phase 1 and Phase 2 enzymes played a major role in determining the outcome of the interaction of cells with carcinogens (2, 7, 20). A wealth of evidence from many laboratories supports the validity of these suggestions (20).

The wide range of chemical agents that evoke the electrophile counter-attack is exemplified in this paper by the effects on liver enzyme profiles of two other xenobiotics: 1,2-dithiole-3-thione, which belongs to a class of compounds that are recognized chemoprotectors (Fig. 1B) (21), and bromobenzene (Fig. 1C) (22), which has not to our knowledge been tested as a chemoprotector. Thus administration of two agents that appeared to be chemically unrelated to each other and to BHA evokes similar and characteristic alterations in the profiles of xenobiotic-metabolizing enzymes.

Elevations of cellular glutathione levels. It has been recognized for many years that the normally high intracellular GSH levels protect cells against a wide variety of toxic chemicals including electrophiles and reactive oxygen species. These protective processes involve both enzymatic (GSTs, glutathione peroxidase) and nonenzymatic reactions (23, 24). Administration of BHA and other substances that elevate Phase 2 enzymes also raised the levels of GSH in the liver and other mouse tissues (1, 4). Similarly, the feeding of low concentrations of 1,2-dithiole-3-thione raised rat hepatic GSH levels 3-fold (21). With the recognition that many chemoprotectors are electrophiles, these observations initially seemed paradoxical, because electrophiles (e.g., diethyl maleate) have been used extensively to deplete the intracellular GSH levels (25). However, depletion of GSH requires high (i.e. stoichiometric or millimolar) concentrations of such electrophiles. Consequently, the findings of Bannai (26-28) and those of Deneke *et al.* (29) that exposure of fibroblasts, endothelial cells, or peritoneal macrophages to micromolar concentrations of diethyl maleate or other electrophiles resulted in substantial elevations of GSH levels are particularly pertinent. The electrophile concentrations required to raise GSH levels are similar to those that induce Phase 2 enzymes. The increases in GSH have been shown to involve enhanced synthesis of a highly specific, sodium-independent, transport system for anionic cystine (in exchange for glutamate). The intracellular concentration of cysteine (derived from imported cystine) is rate-limiting for GSH synthesis. The electrophiles that were shown by Bannai (26) to enhance cystine transport and to raise GSH levels include cyclohex-2-en-1-one, 3-methylcyclohex-2-en-1-one, ethacrynate, maleate, fumarate, cinn-

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mate, 1,2-epoxyethylbenzene, 1,2-ethoxy-3-(*p*-nitrophenoxy)propane, *p*-nitrobenzyl chloride, and bromosulphophthalein. It is remarkable that the same or very similar electrophiles are also inducers of QR in murine hepatoma cells (30, 31). In connection with findings to be discussed below, it is also of interest that stimuli commonly associated with stress protein synthesis, such as sodium arsenite, cadmium chloride, and hyperoxia also enhanced cystine transport and GSH levels (28).

It may be concluded therefore that low concentrations of electrophiles, or of compounds that are converted to electrophiles by metabolism (e.g., BHA), induce a variety of Phase 2 enzymes, and elevate GSH levels through enhanced precursor transport.

CHEMISTRY OF INDUCERS

Structure-activity studies have provided considerable insight into the chemical requirements for inducer activity and the nature of the chemical signal that evokes the elevations of Phase 2 enzymes and GSH levels. Most of the quantitative information on inducer potency has been obtained by measuring QR levels in Hepa 1c1c7 murine hepatoma cells grown and exposed to these inducers in 96-well plates (30, 32). We have established that the response of QR to compounds in this cell culture system is a reliable predictor of inducer activity for QR, GST, and other Phase 2 enzymes in the liver and peripheral tissues of rodents (5, 33, 34).

The growing number and variety of compounds known to evoke these Phase 2 enzyme inductions include at least seven chemically distinct families of monofunctional inducers, i.e. selective inducers of Phase 2 enzymes (6, 30, 31).

(1) *Diphenols, phenylenediamines, and quinones.* From studies of the induction of QR and GST in murine liver by a series of 1,4-dialkyl phenols related to BHA, it was concluded that induction depended on conversion of these compounds to 1,4-diphenols such as *tert*-butylhydroquinone (35). These findings raised the issue whether the 1,4-disposition of the hydroxyl groups was essential for inducer activity. Extensive comparisons of the inducer potencies of 1,2-diphenols (catechols), 1,3-diphenols (resorcinols), and 1,4-diphenols (hydroquinones) demonstrated conclusively that only the 1,2- and 1,4-diphenols were inducers of Phase 2 enzymes, whereas the 1,3-diphenols were always inactive (33, 36). The presence or absence of alkyl substituents on the aromatic nuclei played a relatively minor role in determining inductive potency. Analogous experiments with phenylenediamines demonstrated similar structural requirements, i.e. only the 1,2- and 1,4-phenylenediamines were inducers, but the 1,3-phenylenediamines were not. These findings led to the conclusion that oxidative lability was essential for inducer activity since catechols and hydroquinones (and

their phenylenediamine analogs) undergo facile oxidations to quinones, whereas 1,3-diphenols and 1,3-phenylenediamines cannot participate in such oxidations. Although these experiments did not establish whether the oxidation products or the oxidation processes (potentially involving multiple one- and two-electron oxido-reductions and the generation of reactive oxygen species) were the inductive signal, subsequent work showing the important role of Michael reaction acceptors as inducers strongly suggested that the quinone (or quinoneimine) oxidation products were the ultimate inducers, since these products are strongly electrophilic (30, 31).

(2) *Michael reaction acceptors.* The recognition that many inducers were compounds containing olefins or acetylenes conjugated to electron-withdrawing groups was a major advance in understanding the nature of the inducer signal (30, 31). Such inducers include olefins conjugated with aldehydes, ketones (including thioketones and quinones), esters (e.g., acrylates, crotonates and cinnamates, as well as lactones), nitriles, and nitro groups, i.e. Michael reaction acceptors. The potencies of these compounds as inducers were generally related to the avidities of the electron-withdrawing functions and paralleled the efficiencies of the compounds as Michael reaction acceptors. The concentration dependence of the induction of QR in murine hepatoma cells by a number of selected Michael reaction acceptors is shown in Figure 2.

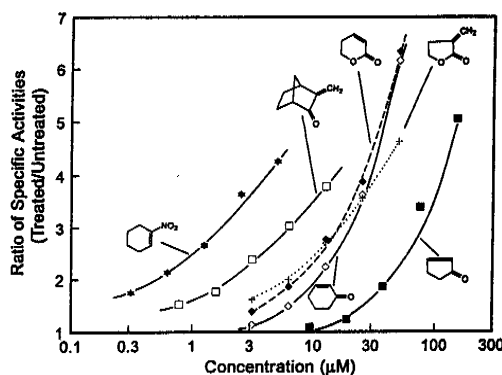


FIG. 2. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of Michael reaction acceptors. The increases in the specific activities of QR are expressed as the ratios of values obtained from treated to untreated cells. Assays were performed in microtiter plates by slight modifications (6, 30) of the method of Prochaska and Santamaria (32). The inducers shown are identified as Nos 1-6 in Figure 7.

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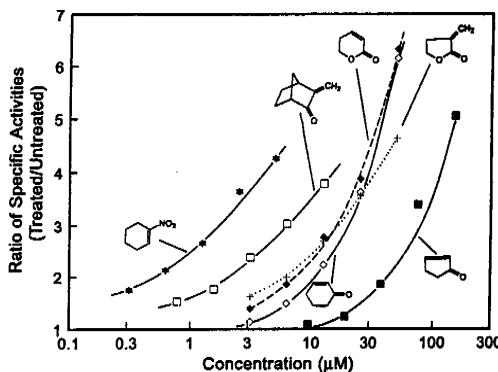


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(3) *Isothiocyanates* ($R-N=C=S$). These compounds are widely distributed in plants and their seeds, where they are often present in the form of glucosinolates (thioglucoside, *N*-hydroxysulfate derivatives) and are accompanied by thioglucoside hydrolases (myrosinases) that convert these compounds to isothiocyanates, HSO_3^- , and glucose (37). The inducer potencies of isothiocyanates vary widely; only those possessing at least a single hydrogen on the α -carbon atom are inducers (e.g., *tert*-butyl and phenyl isothiocyanates are inactive) (30). Sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane: $CH_3-SO-(CH_2)_4-N=C=S$], isolated from broccoli and other crucifers, is a very potent inducer ($CD = 0.21 \mu M$) (10). The responses of QR in murine hepatoma cells to sulforaphane and several other isothiocyanates are shown in Figure 3.

(4) *Hydroperoxides*. Cumene hydroperoxide ($CD = 210 \mu M$) and *tert*-butyl hydroperoxide ($CD = 140 \mu M$), as well as extremely high concentrations of hydrogen peroxide ($CD = 560 \mu M$) are also inducers (6, 38). Whereas the hydroperoxides are substrates for GST, hydrogen peroxide is not thought to be a substrate for these transferases, although it is not clear that it has been tested at the high concentrations required to achieve inductions.

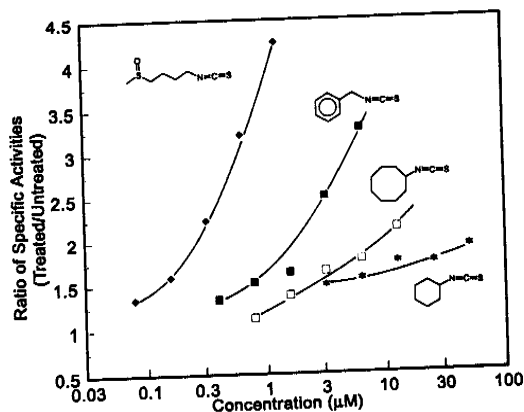


FIG. 3. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of isothiocyanates. The specific activities of QR are presented and were obtained as described in Fig. 2. The inducers are: sulforaphane ($CD = 0.21 \mu M$), and benzyl ($CD = 1.9 \mu M$), cyclooctyl ($CD = 10 \mu M$), and cyclohexyl ($CD = 50 \mu M$) isothiocyanates.

(5) *Mercaptans*. Vicinal dimercaptans such as 2,3-dimercaptopropanol ($CD = 13 \mu M$) and 1,2-ethanedithiol ($CD = 34 \mu M$) were reasonably potent inducers of QR in murine hepatoma cells (Table 1). This was an unexpected finding since most previously known inducers were electrophiles. *Meso*-2,3-dimercaptosuccinic acid ($CD = 120 \mu M$) and its dimethyl ester ($CD = 26 \mu M$) were also inducers. However, the monothiol 2-mercaptoethanol ($CD = 220 \mu M$) was a much weaker inducer, and non-vicinal dithiols such as 1,4-dithiothreitol and 1,4-dithioerythritol were inactive. The relatively high potency of the vicinal mercaptans as inducers is difficult to reconcile with the other structure-activity results. Dimercaptopropanol and dimercaptosuccinates are widely used as metal chelators in the treatment of heavy metal poisonings. These compounds also react with sulfhydryl groups. Whether the redox properties, the metal chelation properties, the ability to react with mercaptans, or some other property of dimercaptans is responsible for the induction of Phase 2 enzymes is currently unclear.

TABLE 1. INDUCTION OF QUINONE REDUCTASE (QR) IN HEPA 1c1c7 CELLS BY MERCAPTANS

Compound	Concentration required to double QR specific activity (μM)
2,3-Dimercaptopropanol (BAL)	$12.9 \pm 3.8^* (N = 12)$
1,2-Ethanedithiol	$33.6 \pm 9.8 (N = 6)$
1,2-Propanedithiol	20
2-Mercaptoethanol	220
<i>meso</i> -2,3-Dimercaptosuccinic acid	120
Dimethyl <i>meso</i> -2,3-Dimercaptosuccinate	26
Dithiothreitol	In†
Dithioerythritol	In
1,4-Butanedithiol	In

* \pm S.D.

†Less than 20% increase at $100 \mu M$.

(6) *Arsenicals*. Trivalent arsenic derivatives are excellent inducers of QR in Hepa 1c1c7 cells (Fig. 4). The hydrophobic phenylarsine oxide ($CD = 0.057 \mu M$) is one of the most potent inducers so far uncovered, and is considerably more potent than sodium arsenite ($CD = 2.1 \mu M$). Sodium arsenate, a pentavalent arsenical, was much less active ($CD = 12 \mu M$). The greater potency of the trivalent arsenicals correlates well with their much higher reactivity with vicinal (or closely-spaced) sulfhydryl groups, and the greater facility with which trivalent arsenicals enter cells (39). These findings are also consistent with the notion that pentavalent arsenic

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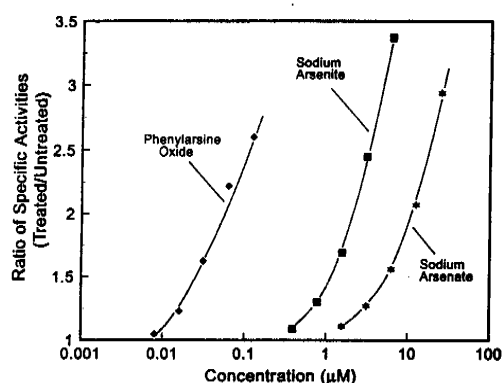


FIG. 4. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of arsenic derivatives. The specific activities of QR are presented and were obtained as described in Fig. 2. The arsenicals are: phenylarsine oxide (No. 26 in Fig. 7; $CD = 0.057 \mu M$); sodium arsenite (No. 27; $CD = 2.1 \mu M$), and sodium arsenate ($CD = 12 \mu M$).

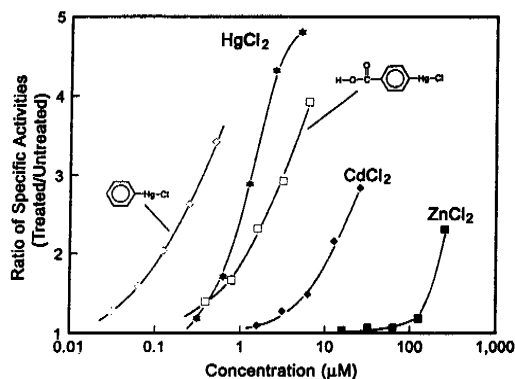


FIG. 5. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of metal derivatives. The specific activities of QR are presented and were obtained as described in Figure 2. The inducers are: phenylmercuric chloride (No. 31 in Fig. 7; $CD = 0.1 \mu M$); $HgCl_2$ ($CD = 0.76 \mu M$); p -chloromercuribenzoic acid (No. 32; $CD = 1.2 \mu M$); $CdCl_2$ ($CD = 10.5 \mu M$); and $ZnCl_2$ ($CD = 230 \mu M$).

derivatives must undergo cellular reduction in order to exert biological (chemotherapeutic or toxic) activity (40). Trivalent arsenicals are classical sulfhydryl reagents that form covalent heterocyclic adducts with vicinal or adjacent sulfhydryl groups (see review in (41)). The potent induction of QR by such compounds suggests the critical presence of two neighboring sulfhydryl groups on the protein(s) that receive and transmit the inductive signal.

(7) *Heavy metals.* The potencies of HgCl_2 , CdCl_2 , and ZnCl_2 as inducers of QR in murine hepatoma cells correlated with their affinities for sulfhydryl groups (Fig. 5). HgCl_2 ($CD = 0.76 \mu\text{M}$) was much more potent than CdCl_2 ($CD = 10.5 \mu\text{M}$), whereas ZnCl_2 ($CD = 230 \mu\text{M}$) was only weakly active. Cadmium and mercury are soft electrophiles which are ideal reagents for thiol groups as these are soft nucleophiles. It may therefore be of special significance that phenylmercuric chloride ($CD = 0.1 \mu\text{M}$) and *p*-chloromercuribenzoate ($CD = 1.2 \mu\text{M}$) which were designed as sulfhydryl reagents are both potent inducers.

MOLECULAR REGULATION OF ELECTROPHILE COUNTERATTACK RESPONSE

Recently, deletion analyses of the 5'-upstream regulatory regions of two Phase 2 enzyme genes: the Ya GST gene (of mouse and rat liver) and the QR gene (of human and rat liver), have been carried out by transient gene expression assays with the use of chloramphenicol acetyltransferase (CAT). The upstream regions of the mouse and rat liver GST Ya genes contain very similar enhancer sequences located within a 41-bp region. These similar, but not identical, enhancer sequences have been designated the Electrophile Responsive Element (EpRE) in the mouse (42) and the Antioxidant Responsive Element (ARE) in the rat (43, 44) GST Ya genes, respectively. The 41-nucleotide DNA segment is located between bp -714 and -754 in the mouse, and -682 and -722 in the rat from the transcription start (Fig. 6). In the mouse, the critical DNA sequences responsive to the few monofunctional inducers tested appear to be the two tandem TGACAT/AT/AGC regions separated by a 6-bp sequence. These two regions have been likened to AP-1 sites, and evidence has been obtained for their functioning in this capacity (45). In the rat GST Ya gene, the single enhancer sequence has been deduced to have the consensus: 5'-puGTGACNNNGC (44). Similar enhancer sequences have also been identified in the upstream regulatory regions of the rat and human quinone reductase genes (45, 47). Only very few of the wide variety of inducers described above had been examined with these transient gene expression constructs. Consequently we recently undertook a comprehensive comparison of the potencies of

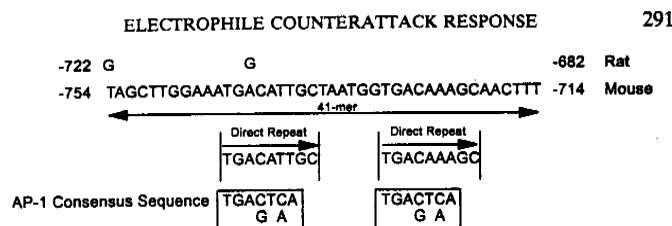


FIG. 6. The homologous 41-bp enhancer sequences from the upstream regions of the mouse and rat glutathione transferase Ya genes (42-44). The lower sequence represents the mouse enhancer region (-714 to -754) and the two base differences in the rat 41-bp sequence (-682 to -722) are shown above. This 41-bp sequence contains two direct semiconserved repeats which are homologous to AP-1 sites (45).

many of the Phase 2 type of inducers in enhancing QR induction in Hepa 1c1c7 murine hepatoma cells, and in stimulating transient gene expression in Hep G2 cells transfected with constructs containing the 41-bp mouse enhancer sequence and the promoter regions of the GST Ya gene (6). Instead of using CAT as the reporter gene, we chose to make constructs expressing human growth hormone (48) so that large numbers of compounds could be screened rapidly and quantitatively.

The concentration required to double the expression of the growth hormone reporter gene (CD_{GH}) and the concentrations required to double the quinone reductase specific activity (CD_{QR}) were compared for 28 compounds belonging to all of the above classes. A plot of the potencies showed a reasonably linear correlation over nearly four orders of magnitude of concentrations varying from phenylarsine oxide ($CD_{GH} = 47$ nM; $CD_{QR} = 57$ nM) to hydrogen peroxide ($CD_{GH} = 210$ μ M; $CD_{QR} = 560$ μ M) (Fig. 7). In addition, six compounds were inactive in both assays (Nos 8, 9, 13, 17, 22, 25). These findings demonstrate that this enhancer element(s) is responsive to all of the chemically diverse types of inducers.

SUMMARY

Exposure of rodents or their cells in culture to low doses of a wide variety of chemical agents, many of which are electrophiles, evokes a coordinated metabolic response that protects these systems against the toxicity (including mutagenicity and carcinogenicity) of higher doses of the same or other electrophiles. This response involves enhanced transcription of Phase 2 enzymes: glutathione transferases, NAD(P)H:quinone reductase, UDP-glucuronosyltransferases, and epoxide hydrolase, as well as the elevation of intracellular levels of reduced glutathione. We suggest that

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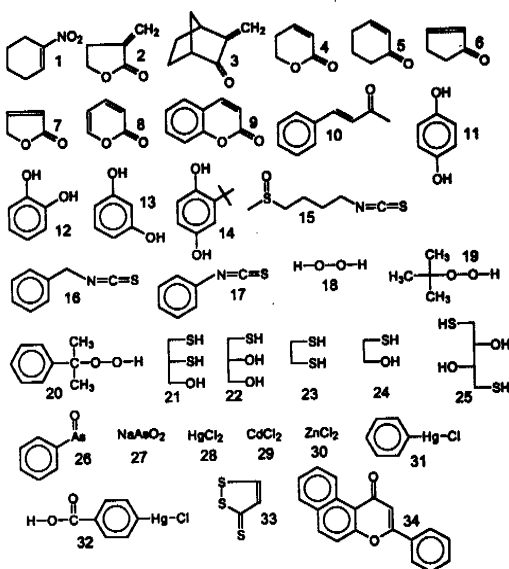
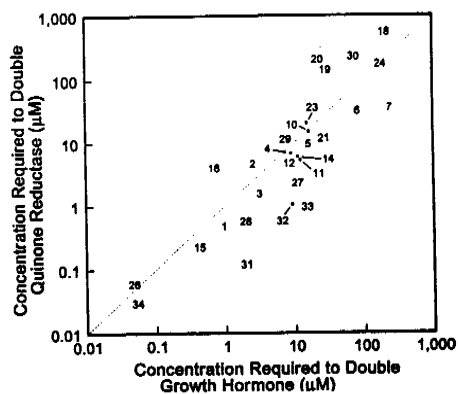
T. PRESTERA, *et al.*

FIG. 7. Caption opposite.

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this cellular adaptation, which occurs in the liver and many peripheral tissues, be designated as the "Electrophile Counterattack" response. Seven families of highly diverse chemical agents that elicit this response include: oxidatively labile diphenols and quinones; Michael reaction acceptors (olefins conjugated to electron-withdrawing groups); isothiocyanates; organic hydroperoxides; vicinal dimercaptans; trivalent arsenicals; heavy metals (HgCl_2 , CdCl_2) as well as mercury derivatives with high affinities for sulfhydryl groups; and 1,2-dithiole-3-thiones. An analysis of the molecular mechanisms of these enzyme inductions was carried out by transient expression in hepatoma cells of a plasmid containing a 41-bp enhancer element derived from the 5'-upstream region of the mouse glutathione transferase Ya gene, and the promoter region of this gene, linked to a human growth hormone reporter gene. The concentrations of 28 inducers (belonging to the seven chemical classes) required to double growth hormone production in this system spanned a range of four orders of magnitude and were closely and linearly correlated with the concentrations of the same compounds required to double the specific activity of quinone reductase in murine hepatoma cells. We therefore conclude that the regulation of these Phase 2 enzymes (and possibly also that of glutathione synthesis) by all of these inducers is mediated by the same enhancer element that contains AP-1-like sites. Similar enhancer sequences are present in the rat glutathione transferase Ya gene, and in the upstream regulatory regions of the quinone reductase genes of rat and human liver.

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FIG. 7. Comparison of potencies of 28 compounds as inducers of quinone reductase and stimulators of growth hormone production in a transient gene expression assay. A total of 34 compounds were tested both for their ability to induce QR in Hepa 1c1c7 cells and for their ability to increase transcription in Hep G2 cells of a reporter gene linked to the 41-bp EpRE element from the mouse GST Ya upstream region (6). Twenty-eight of the compounds tested were active in both assays. These potencies are plotted according to the concentration of each compound required to double the quinone reductase specific activity (ordinate) and the concentration of compound required to double the expression of the growth hormone reporter construct (abscissa). The numbers on the graph correspond to the structures shown below. Compound Nos 8, 9, 13, 17, 22, and 25 were inactive and are not included.

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SCREENING OF PHILIPPINE MEDICINAL PLANTS FOR ANTICANCER AGENTS USING CCNSC PROTOCOLS^{1,2}Victoria A. Masilungan,³ S. Vadlamudi,⁴ and Abraham Goldin⁵

SUMMARY

Extracts of 7 species of plants used locally in the Philippines to treat cancer were screened for the presence of antitumor activity in leukemia L1210, leukemia P388, Sarcoma 180, Adenocarcinoma 755, and Walker carcinosarcoma 256 (intramuscular). Although treatment with extracts of all of the plants resulted in some inhibition of growth in one or more of the tumor systems, none of the plant extracts met the criteria for effectiveness established for these screens in the program of the Cancer Chemotherapy National Service Center.

The Cancer Chemotherapy National Service Center (CCNSC) has published 13 reports (1) dealing with plant extracts which failed to demonstrate sufficient activity in one or more primary screening systems to warrant additional investigation. In the most recent report of Abbott et al (1) almost all of a variety of plant extracts were tested against Sarcoma 180 (S180), Adenocarcinoma 755 (Ca755), leukemia L1210 (L1210), and KB cells in culture. Some tests were also done with Dunning ascites leukemia, Lewis lung carcinoma, Walker carcinosarcoma 256 (intramuscular), human sarcoma HS1, Friend virus leukemia, P1798 lymphosarcoma, and Murphy-Sturm lymphosarcoma. In these screening studies the extracts were derived from plants collected from many parts of the world; however, no collections were made from the Philippines. Nonetheless, some of the extracts used came from the same species of plants reported by Masilungan et al (2) to possess some anticancer activity against Ehrlich ascites tumor cells. Since the constitution of a plant may be influenced by alterations in geographic locale including attendant variations in soil, water, temperature, sunlight, etc, it was considered of interest to conduct additional screening tests with extracts from Philippine medicinal plants listed by Quisumbing (3), and used locally against a variety of malignant diseases.

In the present study the extracts of Philippine medicinal plants were tested for their inhibitory effect on L1210, S180, Ca755, Walker carcinosarcoma 256 (Walker 256) inoculated intramuscularly (im), and leukemia P388 (P388).

MATERIALS AND METHODS

The 7 species of plants listed in table 1 were collected in San José, Batangas, Philippines. The selection of solvent for preparing the extracts was based on the studies of Masilungan et al (2).

Alcoholic extracts of fresh leaves of *Cinnamomum zeylanicum*, *Vitex negundo*, or *Aristolochia tagala* were prepared by macerating 1 kg of ground leaves of each plant in 4 liters of alcohol. After standing for 24 hours at room temperature the extract was filtered and the filtrate evaporated at low temperature to a syrupy consistency. Extracts from the leaves of *Erythrina variegata* Linn. var. *orientalis* and seeds of *Cucumis melo* were also prepared using the above procedure except that 1% HCl was used instead of alcohol. For the leaves of *Viola odorata*, 1% sodium bicarbonate was used instead of alcohol in preparing the extract. An ether extract from the seeds of *Raphanus sativus* was prepared using a Soxhlet extraction apparatus. The ether was then evaporated leaving an oily extract.

In the preparation of an injectable form, vehicles used by the CCNSC were tested as possible diluents for each extract. Selection of the vehicle for each extract (table 1) was based on its capacity for dissolving or suspending the extract. The concentrations of prepared extracts for injection were computed on the basis of moisture-free samples. Alkaline or acid solutions or suspensions of the extracts were adjusted to pH 6-9.

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The 5 transplantable tumor systems used for screening the extracts (L1210, S180, Ca755, Walker 256, and P388) have been used extensively at the CCNSC. The detailed CCNSC protocols were used for each tumor system (4).

Tumor transplantation was done under aseptic conditions. The tumor fragments and tumor cell suspensions were tested for sterility in each experiment using tubes of thioglycollate broth.

The details of the treatment procedures for each test are included with each line of summary data in table 1. The materials were injected either intraperitoneally (ip) or subcutaneously (sc). The dosage schedule involved one injection daily but the number of days of treatment varied with the test system.

The solid tumor systems were evaluated by measurement of tumor weight. The tumor weights are reported in grams for Walker 256 and in milligrams for S180 and Ca755. The ratio of the mean weight of the tumors in treated animals to that in controls ($T/C \times 100 = X$ percent) and the difference in mean body weight change of the animals in the treated and control groups is reported in table 1 for all groups with more than 65% survivors. For these tumors the second stage of sequential testing is done if the ratio of T/C of the first stage is at least equal to 0.44 for S180, Ca755, and Walker 256. For L1210 and P388, the mean survival time of the animals is calculated. The ratio of the mean survival time of the treated group to that of the control groups expressed in percent ($T/C \times 100$) and the difference in mean body weight change between Day 1 and Day 5 of the animals in the treated and control groups was determined for groups with more than 65% survivors on Day 5. With these tumors the second stage of sequential testing is done if the ratio T/C of the first stage is ≥ 1.25 .

The deaths were recorded for all groups. The maximum tolerated dose in an individual experiment is defined as the highest dose which produces not more than 2 deaths in 6 animals or not more than 3 deaths in 10 animals. With L1210, deaths before Day 6 are considered nonleukemic and form the basis for toxic evaluation. When a toxic result ($> 2/6$ or $3/10$ deaths) was observed, the test was repeated at an appropriately lower dose until the maximum tolerated dose was reached. If the T/C value in survival studies was less than 85% the dose was considered too high and was reduced in the next test.

The CCNSC quality control (4) was followed in the experiments. This includes the limitations of toxic deaths, the number of "no takes," and the mean tumor weight range (or survival time range) among control animals. It also includes the use of a positive control.

RESULTS AND DISCUSSION

The results of the screening of 7 species of medicinal plants used in the Philippines for the treatment of cancer are shown in table 1. The data are summarized according to the basic format of Abbott et al (1). In confirmation of previous screening of these species of plants collected in other areas, none of the plants collected in the Philippines met the CCNSC criteria of effectiveness for the L1210, P388, S180, Ca755, and Walker 256 screens; however, at the various dose levels tested extracts of several of the plants did show some indication of tumor inhibitory activity.

Extracts of *A. tagala* at a dose of 500 mg/kg injected ip and 500 and 1000 mg/kg injected sc yielded approximately a 10% increase in survival time in mice that had L1210. There was a slight decrease in the survival time of mice inoculated with P388 when treatment was given at 500 mg/kg, whereas at the same dose level there was 21% and 13% inhibition of tumor growth with S180 and Walker 256 respectively. As indicated by the weight change of the animals and the numbers of survivors of toxicity it is possible that higher doses of the extract might have been used.

Extracts of *C. zeylanicum* at dose levels up to 500 mg/kg were ineffective against L1210 and P388. At a dose of 250 mg/kg it inhibited S180 by 30% and Ca755 by 35%. The inhibitory effect against Walker 256 at the same dose was 19%. The extract appeared to be relatively nontoxic and higher doses might have been used.

Extracts of *C. melo* yielded only an 11% increase in survival time of mice with L1210 and were ineffective in the P388 and Ca755 systems. A dose level of 500 mg/kg resulted in a 22% reduction in tumor weight for S180 and a 30% reduction in tumor weight for Walker 256. The dose level of 500 mg/kg appeared to be somewhat toxic.

Extracts of *E. variegata orientalis* were ineffective in increasing the survival time of mice with L1210 or P388. S180 and Ca755 were inhibited 28% and 23% respectively; Walker 256 showed a 36% reduction in tumor size. Some weight loss in the animals was evident at dose levels of 500 and 1000 mg/kg indicating that maximum tolerated doses had been reached with this extract.

A dose level of 500 mg/kg of the extracts of *R. sativus* had no tumor inhibitory effect against L1210, P388, S180, or Walker 256. With the same dose level there was 28% inhibition of Ca755. A dose level of 1000 mg/kg of the extract increased the survival time of mice that had L1210 by 11%, with some suggestion of toxicity.

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Extracts of *V. odorata* y d only a 10% increase in the survival time of mice L1210 and no increase in the lifespan of mice with P388. They were ineffective against S180 and essentially ineffective against Walker 256 but did yield 32% inhibition for Ca755.

V. negundo yielded an 11% and 5% increase in the survival time of mice with L1210 and P388 respectively and was ineffective against Walker 256. There was 16% inhibition of S180 and 32% inhibition of Ca755.

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Table 1.- In vivo data on plant extracts

Botanical name	Family name	Host*	Test system†	Vehi- cle‡	Route of adminis- tration	Day of injec- tion	No. of injec- tions§	Day anti- mal was killed	Dose (mg/kg, given once daily)	Surv- vors	Wt dif- ference¶ (g)	Tumor wt or survival (T/C)**	Percent (T/C x 100)
<i>Aristolochia tagala</i>	Aristolochiaceae	2	L1210	D	ip	1	Z	died	125	6/6	-0.3	7/7 days	100
		2	L1210	D	ip	1	Z	died	250	6/6	-0.5	7/7 days	100
		2	L1210	D	ip	1	Z	died	500	6/6	-1.3	7.5/7 days	107
		2	L1210	D	sc	3	Z	died	500	6/6	0.4	10/9 days	111
		2	L1210	D	sc	3	Z	died	1000	6/6	-0.2	10/9 days	111
		1	S180	D	ip	1	7	8	500	5/6	-1.6	1046/1330 mg	79
		50	Walker 256	D	ip	3	4	7	500	6/6	-4.4	6.9/7.9 g	87
		2	P388	D	ip	1	10	died	500	6/6		10/11.5 days	87
		2	L1210	S	ip	1	Z	died	125	6/6	-0.9	8.0/8.5 days	94
		2	L1210	S	ip	1	Z	died	150	6/6	-0.8	7.5/8.0 days	94
<i>Cinnamomum zeylanicum</i>	Lauraceae	2	L1210	S	ip	1	Z	died	200	6/6	-0.9	8.0/8.0 days	100
		2	L1210	S	ip	1	Z	died	250	6/6	-3.1	8.5/8.5 days	100
		2	L1210	S	sc	3	Z	died	250	6/6	-2.7	9.0/9.0 days	100
		2	L1210	S	sc	3	Z	died	500	6/6	-0.1	9.0/9.0 days	100
		1	S180	S	ip	1	7	8	250	4/6		780/1110 mg	70
		2	Ca755	S	ip	1	11	12	250	9/9	0.2	1310/2030 mg	65
		3	Walker 256	S	ip	3	4	7	250	6/6	0.2	4.4/5.4 g	81
		2	P388	S	ip	1	10	died	250	6/6		11/12 days	95
		2	L1210	S	ip	1	Z	died	250	6/6	-0.8	8.5/8.5 days	100
		2	L1210	S	sc	3	Z	died	250	6/6	-0.7	10/9 days	111
<i>Cucumis melo</i>	Cucurbitaceae	2	L1210	S	sc	3	Z	died	500	6/6	-0.0	10/9 days	111
		1	S180	S	ip	1	7	8	500	6/6		870/1110 mg	78
		2	Ca755	S	ip	1	11	12	500	9/10	-0.9	2070/2030 mg	102
		3	Walker 256	S	ip	3	4	7	500	6/6	-5.2	3.8/5.4 g	70
		2	P388	S	ip	1	10	died	500	6/6		10/12 days	83
		2	L1210	S	ip	1	Z	died	500	6/6	-0.4	8/8 days	100
		2	L1210	S	sc	3	Z	died	500	6/6	-0.2	9/9 days	100
		2	L1210	S	sc	3	Z	died	1000	6/6	-0.4	9/9 days	100
		1	S180	S	ip	1	7	8	500	4/6		800/1110 mg	72
		2	Ca755	S	ip	1	11	12	500	10/10	-1.0	1570/2030 mg	77
<i>Erythrina variegata</i> Linn. var. <i>orient</i>	Leguminosae	3	Walker 256	S	ip	3	4	7	500	6/6	-3.3	3.4/5.4 g	64
		2	P388	S	ip	1	10	died	500	6/6		10/12 days	83

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<i>Raphanus sativus</i>	Cruciferae	2	L1210	6	ip	1	10	died	500	6/6	10/12 days	83
		2	L1210	6	sc	3	Z	died	500	6/6	8.5/9 days	94
		2	L1210	6	sc	3	Z	died	1000	6/6	9/9 days	100
		2	L1210	6	sc	3	Z	died	1000	6/6	10/9 days	111
		1	S180	6	ip	1	7	8	500	6/6	1440/1330 mg	108
		2	Ca755	6	ip	1	11	12	500	10/10	1297/1805 mg	72
		50	Walker 256	6	ip	3	4	7	500	6/6	8.9/7.4 g	121
		2	P388	6	ip	1	10	died	500	6/6	11/11 days	100
<i>Viola odorata</i>	Violaceae	2	L1210	6	ip	1	Z	died	250	6/6	9/9 days	100
		2	L1210	6	sc	3	Z	died	500	6/6	10/9 days	11
		2	L1210	6	sc	3	Z	died	1000	6/6	10/9 days	110
		1	S180	6	ip	1	7	8	500	6/6	1370/1330 mg	103
		2	Ca755	6	ip	1	11	12	500	10/10	1224/1805 mg	68
		50	Walker 256	6	ip	3	4	7	500	6/6	6.7/7.4 g	92
		2	P388	6	ip	1	10	died	500	6/6	10/11 days	91
<i>Vitex negundo</i>	Verbenaceae	2	L1210	6	ip	1	Z	died	500	6/6	9/9 days	100
		2	L1210	6	sc	3	Z	died	500	6/6	10/9 days	111
		2	L1210	6	sc	3	Z	died	1000	6/6	10/9 days	111
		1	S180	6	ip	1	7	8	500	6/6	1120/1330 mg	84
		2	Ca755	6	ip	1	11	12	500	10/10	1231/1805 mg	68
		50	Walker 256	6	ip	3	4	7	500	6/6	7.8/7.4 g	105
		2	P388	6	ip	1	10	died	500	6/6	11.5/11 days	105

*1 = Swiss mice; 2 = BDF₁ mice; 3 = Sprague-Dawley rats; 50 = random bred albino rats.

†Ca755 = Adenocarcinoma 755; L1210 = lymphoid leukemia L1210; S180 = Sarcoma 180; Walker 256 = Walker carcinosarcoma 256 (im) P388 = Leukemia P388.

‡S = Aikali diluted with saline; 6 = corn oil; D = alcohol.

§Z = received injections until death.

||Number of animals surviving out of number started on tests as defined in individual protocols.

¶Average weight change of treated host minus average weight change of control host (exclusive of tumor weight).

**Tumor weight (mg or g): mean tumor weight of test animals (T)/mean tumor weight of control animals (C).

Survival (days): mean or median survival time of test animals/mean or median survival time of control animals.

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